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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/10, 15/86

A1

(11) International Publication Number: WO 95/29993

(43) International Publication Date: 9 November 1995 (09.11.95)

(21) International Application Number: PCT/US95/05174

(22) International Filing Date: 25 April 1995 (25.04.95)

(30) Priority Data:
08/234,990 28 April 1994 (28.04.94) US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: GENE DELIVERY VECTOR USING PLASMID DNA PACKAGED INTO AN ADENOVIRUS AND A PACKAGING CELL LINE

(57) Abstract

This invention provides a novel expression vector useful for inserting and expressing foreign nucleic acid molecules in a host cell. The expression vector of this invention is derived from an adenovirus and has as its components the adenoviral Inverted Terminal Repeat, an adenoviral packaging sequence, and the DNA molecule to be inserted. This invention also provides a pseudo-adenoviral expression vector having a foreign or heterologous DNA molecule inserted within adenoviral capsid proteins. These vectors are useful for gene therapy.

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GENE DELIVERY VECTOR USING PLASMID DNA PACKAGED INTO AN ADENOVIRUS AND A PACKAGING CELL LINE

This invention was made with government support under grant no. U01 AI 33355 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

A variety of different gene transfer approaches are available to deliver recombinant genes into cells and tissues. Among these are several non-viral vectors, including DNA/liposome complexes, DNA, and targeted viral protein DNA complexes. Several viral vectors, including adenoviruses, adeno-associated viruses, retroviruses, and others have previously been well-described. Most viral vectors have several limitations, including possible biohazard from possible recombination with wild-type vectors, low viral titer and low expression levels. Adenoviral vectors, in contrast, are an effective means for introducing genes into tissues *in vivo* because of their high level of expression and efficient transformation of cells both *in vitro* and *in vivo*, see Davidson, et al., Nature Genetics, 3:219-223 (1993), Quantin, et al., P.N.A.S., 89:2581-2584 (1992) and Mastrangeli, et al., J. Clin. Invest. 91(1):225-34 (1993). However, these viral vectors are disadvantageous for clinical use for two reasons. Because of their ability to recombine with endogenous viruses, adenoviral vectors have a potential for the spread of the recombinant gene in an uncontrolled fashion through the population. In addition, current vectors express multiple viral genes which can be cytopathic and/or immunogenic, yet are not necessarily required for the vector. Thus, a need exists for a vector or gene delivery system which is safe and effective for clinical use. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides a novel expression vector useful for inserting and expressing foreign nucleic acid molecules in a host cell. The expression vector of this invention is derived from an adenoviral vector and has as its components the adenoviral Inverted Terminal Repeat, an adenoviral packaging sequence, and the DNA molecule to be inserted. This invention also provides an adenoviral expression vector having a foreign or heterologous DNA molecule inserted within adenoviral capsid proteins. These vectors are useful for gene therapy.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 graphically depicts a strategy for introducing plasmid DNA into adenoviral particle. The inverted terminal repeat (ITR) packaging sequence of the virus is introduced into a plasmid in such a fashion that the plasmid can be linearized and co-transfected with a mutant full-length virus. The production of viral proteins occurs and allows the plasmid DNA to be packaged in the particle.

Figure 2 shows a segment of adenoviral DNA subcloned into a cosmid vector and linearized before co-transfection into the packaging cell line.

Figure 3 shows the use of a packaging plasmid with the packaging site deleted, but the ITR sequence maintained viral genomic DNA.

Figure 4 schematically [illegible]ts purification and cloning of adenoviral type 5, wi[illegible]nd sub 360 genomic DNA.

Figure 5 is a restriction map of plasmid Psi RSV beta-gal.

Figure 6 is a restriction map of RSV beta-gal.

Figure 7 is a restriction map of plasmid Psi RSV beta-gal-2.

Figure 8 is a restriction map of plasmid Psi RSV beta-gal after partial digestion with AatII, treated with Klenow fragment and created a unique Xba I site.

Figure 9 is a restriction map of the cosmid vector Cos Psi RSV beta-gal.

Figure 10 is a restriction map of packaging plasmid Psi RSV beta-gal LS.

Figures 11A through 11C are restriction maps of cosmid vectors. Figure 11A is the cosmid Psi RSV beta-gal A2. Figure 11B is the cosmid Psi RSV beta-gal S2 and Figure 11C is the cosmid Psi RSV beta-gal AS2.

Figures 12A through 12C are the maps of the adenoviral expression vectors of this invention. Figure 12A is the map of Psi RSV beta-gal LSA2. Figure 12B is the restriction map of Psi RSV beta-gal LSS2 and Figure 12C is the restriction map of Psi RSV beta-gal LSAS2.

DETAILED DESCRIPTION OF THE INVENTION

An object of this invention is to provide adenoviral vectors which can be grown to high titer and infect efficiently. These vectors also are useful for gene because the probability of recombination with is extremely low and they express no adeno genes. Another object of this

invention is to provide an alternate method for introducing recombinant genes into cells for the purposes of treating disease. This is accomplished through the development of a unique adenoviral vector that contains a plasmid DNA rather than adenoviral DNA. This invention offers an advantage over retroviral vectors and conventional prior art adenoviral vectors because it can be grown to high titer stocks, can infect cells efficiently, and is extremely unlikely to recombine in the population.

This invention provides a pseudo-adenovirus vector comprising, from the 5' end to the 3' end, a DNA molecule corresponding to a first adenovirus Inverted Terminal Repeat, a DNA molecule encoding adenovirus packaging sequence, a heterologous DNA, and a DNA molecule corresponding to a second adenovirus Inverted Terminal Repeat. As used herein, the term "pseudo-adenovirus vector" is intended to include DNA molecules that can be transferred into the host cell in adenovirus capsids to express a recombinant gene. As used herein, the term "expression vector" is intended to mean a vehicle that promotes the expression of a gene inserted into it; typically, a restriction fragment that carries a regulatory sequence for the particular gene and sequences that provide for RNA polyadenylation and processing.

The term "heterologous DNA" is intended to encompass a DNA polymer. For example, the heterologous DNA comprises plasmid vector DNA or cosmid vector DNA. Prior to insertion into the pseudo-adenoviral vector, the heterologous DNA is in the form of a separate fragment, or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the segment and its component nucleotide sequences by standard biochemical methods, for

example, using a cloning vector. As used herein, "recombinant" is intended to mean that a particular DNA sequence is the product of various combination of cloning, restriction, and ligation steps resulting in a construct having a sequence distinguishable from homologous sequences found in natural systems. Recombinant sequences can be assembled from cloned fragments and short oligonucleotides linkers, or from a series of oligonucleotides.

In one aspect of this invention, the pseudo-adenovirus expression vector and the adenovirus capsids are derived from adenovirus type 5 virus. Other suitable adenoviral subtypes are human types 1-41 or murine strains.

In yet another aspect of this invention, the vector further contains a DNA molecule containing adenovirus packaging sequence which allows the genetic material to be assembled and packaged into the adenoviral particle. This sequence is comprised of multiple, (6-20) oligonucleotide repeats derived from sequence 3' to the left ITR (Grable et al.(1990) infra.).

The heterologous DNA also can contain additional DNA molecules which comprise a transcriptional initiation region so that DNA molecules downstream from the initiation region can be transcribed to a sequence of interest, usually mRNA, whose transcription and, as appropriate, translation will result in the expression of a polypeptide, a protein, a ribozyme and/or the regulation of other genes, e.g. antisense, expression of transcriptional factors, etc.

There are technical considerations in introducing adenoviral DNA into adenoviral complexes. the *cis*-acting DNA sequences required for pa are the inverted terminal repeats (ITR), whic for replication of the DNA in cells t gene products. Second, the pre

sequence is required. These sequences have been defined, in part, by deletion analysis of minimal regions required for packaging, and have been previously described (Grable et al., J. Virol. 64:2047-2056 (1990) incorporated herein by reference). Third, the length of the DNA to be packaged within the adenoviral sequence needs to be considered. In the present invention, several means to introduce the recombinant DNA into the adenoviral particle have been set forth.

Conventionally, adenoviral packaging is accomplished using a plasmid containing the left end of the adenoviral genome which is replication defective and co-transfecting with wild type adenoviral DNA inactivated to prevent its replication. In the present application, there are three strategies that have been taken to introduce plasmid DNA into the adenoviral particle. In the first case (Figure 1), the ITR packaging sequence of the virus is introduced into a plasmid in such a fashion that the plasmid can be linearized and co-transfected with the virus DNA. Thus, the production of viral proteins occurs and allows the plasmid DNA to be packaged in the particle. In a variation of this approach (Figure 2), a segment of adenoviral DNA is subcloned into a cosmid vector and linearized before co-transfection into the packaging cell line, thus also allowing for packaging of the recombinant DNA in the transfected cell line. The advantage of this approach is that an artificial form of the truncated virus is used, thus minimizing the possibility that uncut viral DNA will be present in the cell culture and will allow for the replication of wild-type adenovirus. Finally, in the preferred embodiment (Figure 3), the packaging plasmid is used, together with an adenovirus in which the packaging site has been deleted but the ITR sequence is maintained, thus allowing replication of defective virus and viral proteins at the same time that the plasmid DNA is replicated, giving the higher titer

virus. A further development of this technology is a permanent packaging cell line which provides the viral packaging proteins in *trans*, and thus require only the transfection of the plasmid DNA with the packaging sequence within. The present studies demonstrate the feasibility of using a packaging sequence and ITR anti-plasmid to allow incorporation of the DNA into the antiviral particle. The addition of nonviral DNA sequences to further improve efficiency are within the scope of this invention. Other aspects include to introduce adenoviral sequences to further define the other cis-acting regulatory elements required for packaging, and finally, to introduce additional consensus packaging sequences into the background of irrelevant DNA (phage DNA) to further improve the efficiency of packaging of the plasmid vector.

MATERIALS AND METHODS

Cell Culture

The transformed human embryonic kidney cell line, 293, (ATCC) was maintained in Dulbecco's Modified Eagle Medium (D-MEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-Glutamine.

DNA and Plasmid

Purification of Ad5 and sub360 genomic DNA (Figure 4)

For preparation of Adenovirus type 5 wild type and its derivative, sub360 genomic DNA, 293 cells were infected with each virus lysate (10 plaque forming units/cell). The adenovirus particles were purified by CsCl density centrifugation (Graham, et al., Virology 52:456-467 (1973) incorporated herein by reference), then ted wi[illegible] 2 mg/ml of self-digested Pronase E (Sigma) in [illegible] .4, 1mM EDTA and 0.5% SDS solution at 37°C

for 45 min., extracted with phenol-chloroform twice and with chloroform once. Genomic DNA was recovered by ethanol precipitation.

pWEsub360 (Figure 4)

The sub360 DNA was treated with T4 polynucleotide kinase and Klenow fragment to repair the ends of the genomic DNA. Following the ligation of Xba I linkers (Promega) to each end, the genomic DNA was digested with Xba I. The right hand fragment of sub360 was cloned into the Xba I site of cosmid vector pWE15 (Strategene) which was modified by creating a new Xba I site into the BamHI site according to the manufacturer's instructions.

ψRSV βGal (Figures 5, 6)

For cloning of the Ad5 terminal sequence and packaging signal sequence (Grable, et al. (1990) supra.), pAd-Bgl II plasmid (Davidson, et al., Nature Genetics, 3:219-223 (1993) incorporated herein by reference) was digested with Eco RI and repaired by Klenow fragment of *E. coli* DNA polymerase. After ligation of BamHI linkers (Boehringer) to the blunted Eco RI sites, the plasmid was digested with BamHI and BgI II. A DNA fragment containing the terminal sequence and packaging signal sequence (370 bp) was introduced into the BamHI site of RSV βGal (Stewart, et al. Human Gene Therapy, 3:267-275 (1992) incorporated herein by reference). This clone was tentatively coded as Pack+RSV βGal. Another terminal sequence was generated by Polymerase Chain Reaction (PCR) using pAd-Bgl II as a DNA template. In this reaction, the primers were designed as follows: sense primer containing an Eco RI site (nucleotide number of pAd Bgl II 1-29)

5'-ACAGAATTCGCTAGCATCATCAATAATATACC-3', (Seq

and anti-sense primer (200-173) containing a BamHI site, 5'-ACAGGATCCGGCGCACACCAAAAACGTCACTTTTGCC-3' (Seq. I.D. No. 2). The PCR conditions were 94°C 30 seconds; 65°C 30 seconds; and 72°C 30 seconds for the first 5 cycles, then 94°C 30 seconds; and 72°C 30 seconds for 30 cycles. The amplified terminal sequence (212 bp) was digested with Eco RI and BamHI and subcloned into pBluescript (Strategene). Following introduction of a BamHI linker into the Xho I site of this plasmid, the terminal sequence fragment was purified by BamHI digestion, and introduced into the BamHI site of Pack+RSV βGal plasmid to generate an Inverted Terminal Repeat (ITR). The ψ RSV βGal plasmid was propagated in *E. coli*, SURE Cells (Strategene).

pAdΔψ

To construct a pAdΔψ plasmid that encoded the Ad5 left hand DNA sequence, deleted for the packaging signal sequence, the terminal sequence in the above pBluescript plasmid was purified by digestion with Nhe I and BamHI, and cloned into the Nhe I and BgI II sites of pAd Bgl II.

Transfection

Co-transfection was performed by the calcium phosphate method (Sambrook, et al., Molecular Cloning: A Laboratory Manual (1989) Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference) in 100 mm diameter petri dishes, 293 cells were transfected with 10 μg Eco RI digested pAdΔψ, 10μg Nhe I digested ψRSV βGal, and varying amounts of Xba I and Cla I digested sub 360 genome, or Xba I and Klenow fragment-treated pWEsub360. In control experiments, 10μg of BamHI digested RSV βGal was used in place of ψ RSV βGal. Eight hours post-transfection, cells were harvested, suspended in 2 mls of medium and freeze-thawed 3 times in dry ice/ethanol. Supernatants were used as viral lysates in the experiments.

Titration of Virus

Confluent 293 cells in 60 mm diameter dishes were infected with 0.5 ml of viral lysate for 1 hr. After infection, 4.5 mls of medium were overlaid, and cells were cultured for 24 hours at 37°C. The infected cells were harvested, washed with PBS twice, and fixed with 1.25% glutaraldehyde-PBS solution for 5 min. at room temperature. Fixed cells were washed with PBS twice and stained with Solution X [50 mM Tris HCl, pH 7.5, 2.5 mM Ferriferrocyanide, 15 mM NaCl, 1mM $MgCl_2$ and 0.5 mg/ml X-gal] overnight in 6 well culture plates. The number of blue stained cells and total cells in each well were counted (Table 1).

TABLE 1

Adenovirus packaging sequence induces incorporation of linearized plasmid DNA into virus particles - evidence of transduction and expression.

	Vector	Conc.Sub360 (μg)	# Positive cells/plate
Experiment 1	RSV ßGal	0.5	0.9
	ψRSV ßGal		122.1
	RSV ßGal	1.0	26.7
	ψRSV ßGal		230.0
Experiment 2	RSV ßGal	0.5	2.3
	ψRSV ßGal		9.6
	RSV ßGal	1.0	4.1
	ψRSV ßGal		56.6

ß-galactosidase activity of RSV ßGal or ψRSV ßGal co-transfected with sub360 digested with Xba I and Cla I and pAdΔψ (Experiment 1); co-transfected with pWEsub360 and pAdΔψ (Experiment 2).

RSV [illegible]al plasmid (Figure 7) was used as [illegible]nstruct the large-size plasmids.

Psi RSV beta-gal plasmid was partially digested with AatII and treated with Klenow fragment, then an XbaI linker (Progega) was introduced (nucleotide position, 5,775). This plasmid was tentatively named Psi RSV beta-galXbaI (Figure 8).

Separately, a cosmid vector, SuperCos1 (Stratagene) was digested with XbaI and NheI, and blunt-ends created by Klenow fragment incubation. Then, a NotI linker (Promega) was introduced into this position. The cos fragment was prepared by digestion with HinfI and EcoRI and by treatment with Klenow fragment. This fragment (2,371 bp) was inserted into the blunt-ended SalI site of Psi RSV beta-galXbaI, described above. This cosmid vector was coded as Cos Psi RSV beta-gal (Figure 9). For the ligation reaction with yeast or phage λ genomic DNA, Cos Psi RSV beta-gal plasmid was digested NotI, treated with Calf intestinal alkaline phosphatase, then, additionally digested with XbaI. Yeast genomic DNA was completely digested with NheI and treated with alkaline phosphatase. The DNA fragments were separated on 0.5% low melting agarose gel, the fragments ranging 20-30 kb were purified. These fragments were ligated to NotI, XbaI-digested Cos Psi RSV beta-gal plasmid, described above, then, packaged into lambda phage using the Gigapack II packaging kit (Stratagene). The clones, whose total sizes ranged between 20-40kb were selected, and designated packaging plasmids Psi RSV beta-galLS (Figure 10).

To enhance the adenoviral packaging efficiency of these plasmids, another Psi RSV beta-gal LS plasmids also was constructed which had additional packaging signals. The oligonucleotides which coded packaging signal element AV and AVI (Grable and Hearing, J. Virol. 66:723-731 (1992) incorporated herein by reference) were designed as [illegible] Sense primer which had ApaI restriction site at [illegible]

5'-GCGTAATATTTGTCTAGGGCCGCGGGGACTTTGGGGCC-3', (Seq. I.D. No. 3)

anti-sense primer which had ApaI site at 5'-end;

5'-CCAAAGTCCCCGCGGCCCTAGACAAATATTACGCGGCC-3' (Seq. I.D. No. 4).

Sense primer which had SapI site at 5'-end;

5'-GCTCGTAATATTTGTCTAGGGCCGCGGGGACTTTGG-3', (Seq. I.D. No. 5)

anti-sense primer which had SapI site at 3'-end;

5'-AGCCCAAAGTCCCCGCGGCCCTAGACAAATATTACG-3' (Seq. I.D. No. 6).

All 5'-ends of sense and anti-sense oligonucleotides were phosphorylated by T4 polynucleotide kinase and annealed. The oligonucleotides which had either ApaI site or SapI site were introduced into ApaI or SapI site of cos Psi RSV beta-gal to create two (2) tandem copies and also to show the same direction as that of wild-type packaging signal in Cos Psi RSV beta-gal (Figure 11). The plasmid which contained oligonucleotides at ApaI site was called Cos Psi RSV beta-galA2 and the Sap I site was termed Cos Psi RSV beta-galS2. When a plasmid was constructed which contained the oligonucleotides at both ApaI and SapI site, the oligonucleotide which bore the SapI sequence at the end was inserted into SapI site of Cos Psi RSV beta-galA2. This plasmid was named Cos Psi RSV beta-galAS2 (Figure 11C). To increase the total length of Cos Psi RSV beta-galA2, S2 and AS2, NheI digested-yeast genomic DNAs were ligated into XbaI site of [illegible]smids, packaged into lambda phage as previously [illegible] The plasmids which showed those size betwe[illegible] selected. The plasmids generated fro[illegible] were coded

as Psi RSV beta-gal LSA2, from Cos Psi RSV beta-galS2 were Psi RSV beta-galLSS2, and from Cos Psi RSV beta-galAS2 were Psi RSV beta-galLSAS2, as well (Figure 12).

The expression vectors of this invention can be inserted into host cells, for example, mammalian cells, particularly primate, more particularly human, but can be associated with any animal of interest, particularly domesticated animals, such as equine, bovine, murine, ovine, canine, feline, etc. Among these species, various types of cells may be involved, such as hematopoietic, neural, mesenchymal, cutaneous, mucosal, stromal, muscle, spleen, reticuloendothelial, epithelial, endothelial, hepatic, kidney, gastrointestinal, pulmonary, etc. Of particular interest are hematopoietic cells, which can include any of the nucleated cells which may be involved with the lymphoid or myelomonocytic lineages. Also of particular interest are members of the T- and B-cell lineages, macrophages and monocytes. Further of interest are stem and progenitor cells, such as hematopoietic neural, stromal, muscle, hepatic, pulmonary, gastrointestinal, etc.

The heterologous DNA also can code for receptors which may include receptors for the ligands IL-2, IL-3, IL-4, IL-7 (interacts with p59fyn); erythropoietin (EPOR), G-CSF, leukemia inhibitory factor (LIF), ciliary neutryphic factor (CNTR), growth hormone (GH), herpesvirus thymidine kinase, histocompatibility genes, and prolactin (PRL).

The heterologous DNA also may contain DNA sequences which provides for the necessary transcriptional termination, and as appropriate, translational termination.

The heterologous DNA can contain a wide variety of [illegible] the gene encodes a protein of interest or [illegible] of interest. The gene can be any

sequence of interest which provides a desired phenotype. The gene can express a surface membrane protein, a secreted protein, a cytoplasmic protein, or there may be a plurality of genes which may express different types of products. The gene also can encode an antisense sequence which may modulate a particular pathway by inhibiting a transcriptional regulation protein or turn on a particular pathway by inhibiting an inhibitor of the pathway. The proteins which are expressed, singly or in combination, may involve homing, cytotoxicity, proliferation, immune response, inflammatory response, clotting or dissolving of clots, hormonal regulation, or the like. The proteins expressed could be naturally-occurring, mutants of naturally-occurring proteins, unique sequences, or combinations thereof.

The gene also can encode a product which is secreted by a cell, so that the encoded product may be made available at will, whenever desired or needed by the host. Various secreted products include hormones, such as insulin, human growth hormone, glucagon, pituitary releasing factor, ACTH, melanotropin, relaxin, etc.; growth factors, such as EGF, IGF-1, TGF-α, -β, PDGF, G-CSF, M-CSF, GM-CSF, FGF, erythropoietin, megakaryocytic stimulating and growth factors, etc.; interleukins, such as IL-1 to -11; TNF-α and -β, etc.; and enzymes, such as tissue plasminogen activator, members of the complement cascade, perforans, superoxide dismutase, coagulation factors, anti-thrombin-III, Factor VIIIc, Factor VIIIvW, α-anti-trypsin, protein C, protein S, etc.

The gene also can encode a surface membrane protein. Such proteins may include homing receptors, e.g. L-selectin (Mel-14), blood-related proteins, particularly having a kringle structure, e.g., Factor VIIIc, Factor VIIIvW, hematopoietic cell markers, e.g. CD3, CD4, B cell receptor, TCR subunits α, β, γ, δ, CD10,

CD33, CD38, CD41, etc., receptors, such as the interleukin receptors IL-2R, IL-4R, etc., channel proteins, for influx or efflux of ions, e.g., H^+, Ca^{+2}, K^+, Na^+, Cl^-, etc., and the like; CFTR, tyrosine activation motif, zeta activation protein, etc.

Also, intracellular proteins may be of interest, such as proteins in metabolic pathways, regulatory proteins, steroid receptors, transcription factors, etc., particularly depending upon the nature of the host cell. Some of the proteins indicated above may also serve as intracellular proteins.

The following are a few illustrations of different genes. In T-cells, one may wish to introduce genes encoding one or both chains of a T-cell receptor. For B-cells, one could provide the heavy and light chains for an immunoglobulin for secretion. For cutaneous cells, e.g. keratinocytes, one could provide for infectious protection, by secreting α-, β- or γ-interferon, antichemotactic factors, proteases specific for bacterial cell wall proteins, etc.

In addition to providing for expression of a gene which may have therapeutic value, there will be many situations where one may wish to direct a cell to a particular site. The site may include anatomical sites, such as lymph nodes, mucosal tissue, skin, synovium, lung or other internal organs or functional sites, such as clots, injured sites, sites of surgical manipulation, inflammation, infection, etc. By providing for expression of surface membrane proteins which will direct the host cell to the particular site by providing for binding at the host target site to a naturally occurring epitope, localized concentrations of the product may be achieved. Proteins of interest include receptors, e.g. L-selectin, GMP140, [illegible], e.g.

ELAM-1, PNAd, LNAd, etc., clot binding proteins, or cell surface proteins that respond to localized gradients of chemotactic factors. There are numerous situations where directing cells to a particular site, where release of a therapeutic product could be of great value. Among these would be the delivery of a recombinant gene to malignant cells for the purpose of causing cell death or inducing immune recognition of tumors.

An additional example is autoimmune disease. Cells of extended lifetime, e.g. endothelial cells could be employed. The heterologous DNA would provide for a homing receptor for homing to the site of autoimmune injury and for cytotoxic attack on cells causing the injury. The therapy would then be directed against cells causing the injury. Alternatively, one could provide for secretion of soluble receptors or other peptide or protein, where the secretion product would inhibit activation of the injury causing cells or induce anergy. Another alternative would be to secrete an anti-inflammatory product, which could serve to diminish the degenerative effects.

The genes can be introduced in one or more DNA molecules or expression vectors, where there will be at least one marker and may be two or more markers, which will allow for selection of host cells which contain the gene(s). The heterologous DNA, genes and expression vectors can be prepared in conventional ways, where the genes and regulatory regions may be isolated, as appropriate, ligated, cloned in an appropriate cloning host, analyzed by restriction or sequencing, or other convenient means. Particularly, using PCR, individual DNA fragments including all or portions of a functional unit may be [illegible], where one or more mutations may be intr[illegible] "primer repair", ligation, etc. as app[illegible] Sambrook et al. Molecular Cloning: A [illegible] Cold Spring Harbor Press, N.Y.,

incorporated herein by reference. Host cells can be grown and expanded in culture before introduction of the vector(s) followed by the appropriate treatment for introduction of the vectors and integration of the vector(s). The cells will then be expanded and screened by virtue of a marker present in the vector. Various markers which may be used successfully include *hprt*, neomycin resistance, thymidine kinase, hygromycin resistance, etc.

The expression vectors can be introduced simultaneously or consecutively, each with the same or different markers.

Depending upon the nature of the cells, the cells may be administered in a wide variety of ways. Hematopoietic cells may be administered by injection into the vascular system, there being usually at least about 10^4 cells and generally not more than about 10^{10}, more usually not more than about 10^8 cells. The number of cells which are employed will depend upon a number of circumstances, the purpose for the introduction, the lifetime of the cells, the protocol to be used, for example, the number of administrations, the ability of the cells to multiply, the stability of the therapeutic agent, the physiologic need for the therapeutic agent, and the like. Alternatively, with skin cells which may be used as a graft, the number of cells would depend upon the size of the layer to be applied to the burn or other lesion. Generally, for myoblasts or fibroblasts, the number of cells will at least about 10^4 and not more than about 10^8 and may be applied as a dispersion, generally being injected at or near the site of interest. The cells will usually be in a physiologically-acceptable medium.

The vectors of this invention can be used for the treatment of a wide variety of conditions and indications. For example, B- and T-cells, antigen-presenting cells

malignant cells themselves may be used in the treatment of cancer, infectious diseases, metabolic deficiencies, cardiovascular disease, hereditary coagulation deficiencies, autoimmune diseases, joint degenerative diseases, e.g. arthritis, pulmonary disease, kidney disease, nedocrine abnormalities, etc. Various cells involved with structure, such as fibroblasts and myoblasts, may be used in the treatment of genetic deficiencies, such as connective tissue deficiencies, arthritis, hepatic disease, etc. Hepatocytes could be used in cases where large amounts of a protein must be made to complement a deficiency or to deliver a therapeutic product to the liver or portal circulation.

This invention also provides a transgenic, non-human animal whose germ cells and somatic cells contain a heterologous DNA molecule that has been introduced into the animal, or an ancestor of the animal, at an embryonic stage. When the heterologous DNA molecule encodes an product which produces a pathological condition in the animal, these animals are useful to test materials suspected of treating the pathology. Alternatively, the heterologous DNA can be used to encode a therapeutic or prophylactic composition. These animals are useful to test the particular therapy. Using the vectors of this invention and methods well known to those of skill in the art (for example, Leder et al., U.S. Patent No. 4,736,866, issued April 12, 1988, incorporated herein by reference), the transgenic animals can be produced.

Although the invention has been described with reference to the above embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: THE UNIVERSITY OF MICHIGAN

(ii) TITLE OF INVENTION: GENE DELIVERY VECTOR USING PLASMID DNA PACKAGED INTO AN ADENOVIRUS AND A PACKAGING CELL LINE

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: MORRISON & FOERSTER
(B) STREET: 755 PAGE MILL ROAD
(C) CITY: PALO ALTO
(D) STATE: CALIFORNIA
(E) COUNTRY: USA
(F) ZIP: 94304-1018

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/234,990
(B) FILING DATE: 28-APR-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: KONSKI, ANTOINETTE F.
(B) REGISTRATION NUMBER: 34,202
(C) REFERENCE/DOCKET NUMBER: 20344-20910.40

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 813-5600
(B) TELEFAX: (415) 494-0792
(C) TELEX: 706141

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACAGAATTCG CTAGCATCAT CAATAATATA CC
32

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: [illegible] base pairs
(B) TYPE: [illegible] acid
(C) [illegible] gle
(D) [illegible]

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACAGGATCCG GCGCACACCA AAAACGTCAC TTTTGCC 37

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCGTAATATT TGTCTAGGGC CGCGGGGACT TTGGGGCC 38

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCAAAGTCCC CGCGGCCCTA GACAAATATT ACGCGGCC 38

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTCGTAATA TTTGTCTAGG GCCGCGGGGA CTTTGG 36

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCCCAAAGT CCCCGCGGCC CTAGACAAAT ATTACG 36

What is claimed is:

1. A pseudo-adenovirus expression vector, comprising, from the 5' end to the 3' end, a DNA molecule corresponding to a first adenovirus Inverted Terminal Repeat, a DNA molecule encoding adenovirus packaging sequence, a heterologous DNA, and a DNA molecule corresponding to a second adenovirus Inverted Terminal Repeat.

2. The pseudo-adenovirus expression vector of claim 1, wherein the adenovirus capsid is derived from adenovirus type 5 virus.

3. The pseudo-adenovirus expression vector of claim 1, further comprising a second DNA molecule containing adenovirus packaging sequences.

4. The pseudo-adenovirus expression vector of claim 1, wherein the heterologous DNA comprises plasmid vector DNA or cosmid vector DNA.

5. The pseudo-adenovirus expression vector of claim 1, wherein the heterologous DNA further comprises a promoter for transcription.

6. The pseudo-adenovirus expression vector of claim 1, wherein the heterologous DNA codes for a ribozyme, a protein, a polypeptide, or an antisense RNA molecule.

7. A gene expression system comprising the pseudo-adenovirus expression vector of claim 1 and a packaging defective adenovirus helper virus.

8. The gene expression system of claim wherein the defective adenovirus is derived from virus.

9. The gene expression system of claim 7, wherein the adenovirus expression vector further comprising a second DNA molecule encoding adenovirus packaging sequence.

10. The gene expression system of claim 7, wherein the heterologous DNA comprises plasmid vector DNA or cosmid vector DNA.

11. The gene expression system of claim 7, wherein the heterologous DNA further comprises a promoter for transcription.

12. The gene expression system of claim 7, wherein the heterologous DNA codes for a ribozyme, a protein, a polypeptide, or an antisense RNA molecule.

13. A pseudo-adenoviral expression vector comprising a heterologous DNA molecule and adenoviral capsid proteins, the DNA molecule being encapsulated within the capsid proteins.

14. The pseudo-adenovirus expression vector of claim 13, wherein the adenovirus capsid is derived from adenovirus type 5 virus.

15. The pseudo-adenovirus expression vector of claim 13, wherein the heterologous DNA comprises plasmid vector DNA or cosmid vector DNA.

16. The pseudo-adenovirus expression vector of claim 13, wherein the heterologous DNA further comprises a promoter for transcription.

17. The pseudo-adenovirus expression vector of claim 13, wherein the heterologous DNA codes for a ribozyme, a protein, a polypeptide, or an antisense RNA molecule.

18. A host cell comprising the pseudo-adenovirus expression vector of claim 1.

19. A host cell comprising the pseudo-adenovirus expression vector of claim 13.

20. The host cell of claim 18 or 19, wherein the pseudo-adenovirus is derived from adenovirus type 5 virus.

21. The host cell of claim 18 or 19, wherein the heterologous DNA comprises plasmid vector DNA or cosmid vector DNA.

22. The host cell of claim 18 or 19, wherein the heterologous DNA further comprises a promoter for transcription.

23. The host cell of claim 18 or 19, wherein the heterologous DNA codes for a ribozyme, a protein, a polypeptide, or an antisense RNA molecule.

24. A non-human transgenic animal comprising the pseudo-adenoviral expression vector of claim 13.

25. The non-human transgenic animal of claim 24, wherein the pseudo-adenovirus is derived from adenovirus type 5 virus.

26. The non-human transgenic animal of claim 24, wherein the heterologous DNA comprises plasmid vector DNA or cosmid vector DNA.

27 The non-human transgenic animal of claim 24, wh he heterologous DNA further comprises a promoter ription.

28. The non-human transgenic animal of claim 24, wherein the heterologous DNA codes for a ribozyme, a protein, a polypeptide, or an antisense RNA molecule.

29. A method of introducing a heterologous DNA molecule into a cell which comprises inserting into the cell the pseudo-adenovirus expression vector of claim 1.

30. A method of introducing a heterologous DNA molecule into a cell which comprises contacting the cell with the pseudo-adenovirus expression vector of claim 13.

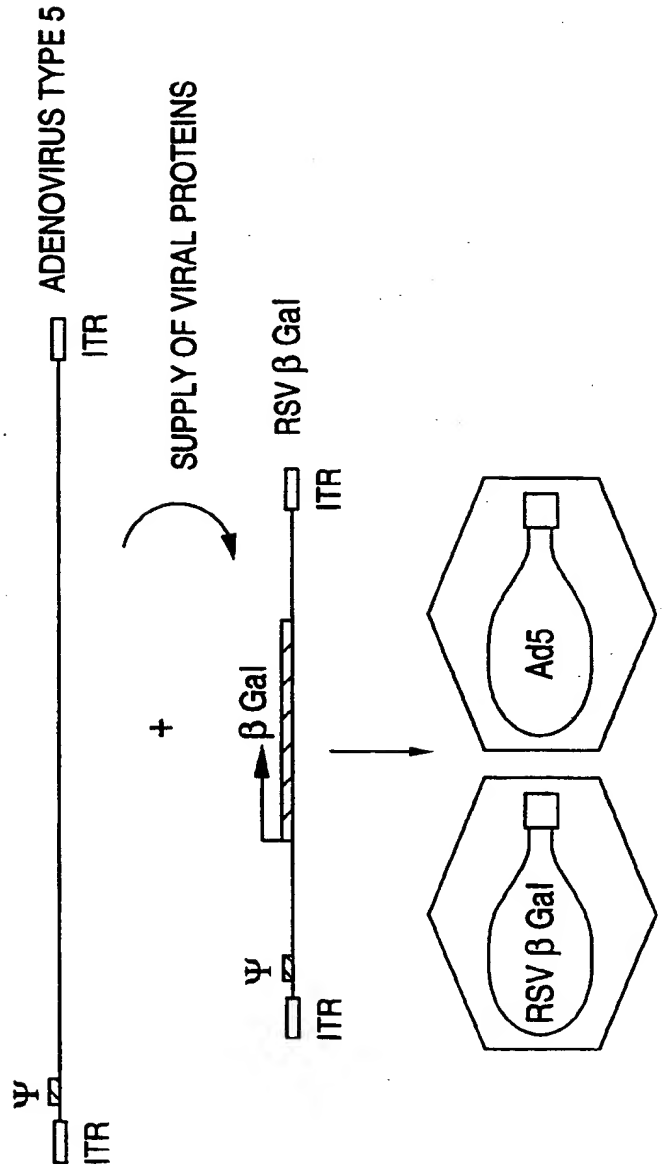

ADENOVIRUS TYPE 5
ITR
Ψ
ITR
SUPPLY OF VIRAL PROTEINS
+
β Gal
RSV β Gal
ITR
Ψ
ITR
Ad5
RSV β Gal

FIG. 1

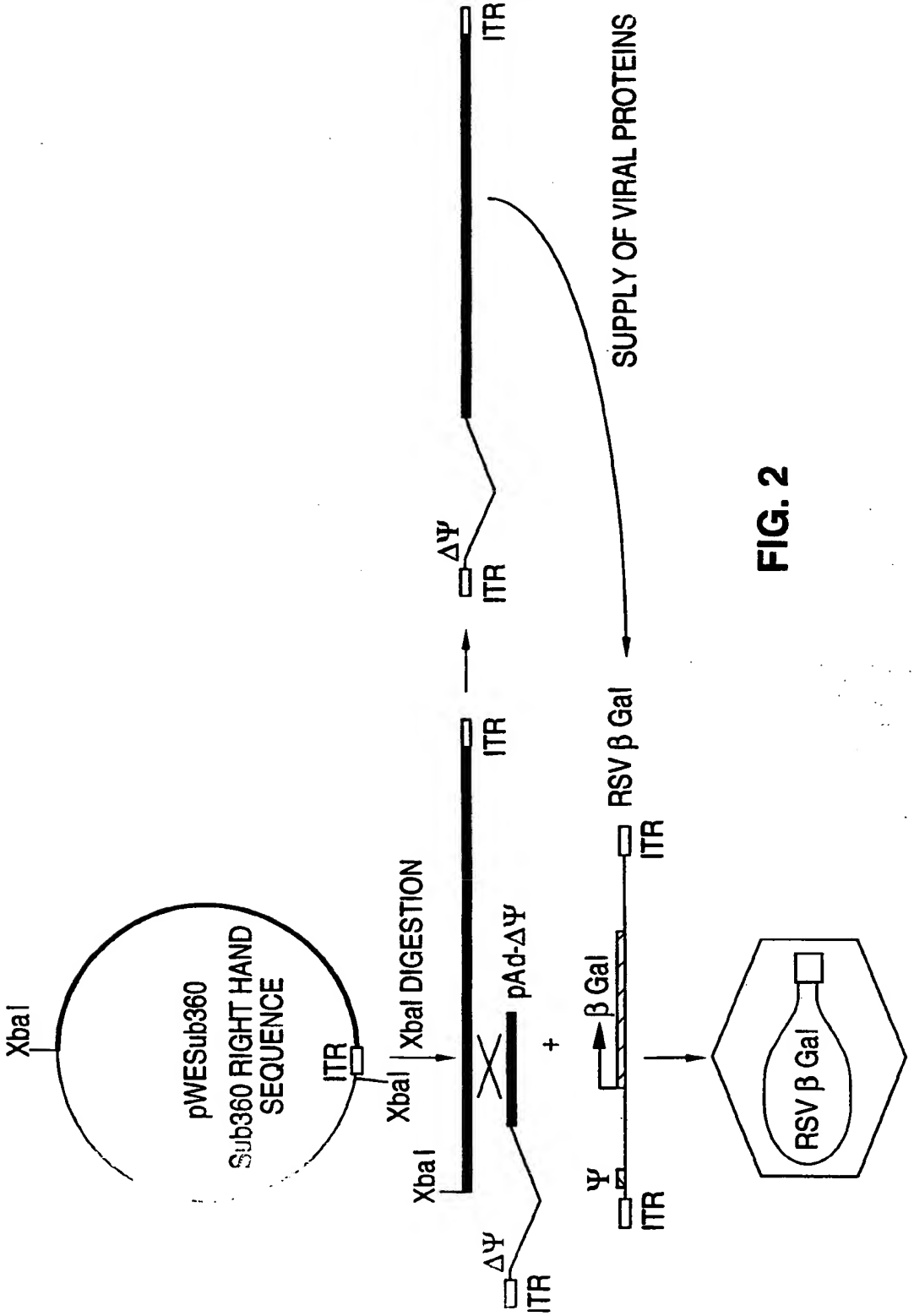

XbaI
pWESub360
Sub360 RIGHT HAND SEQUENCE
ITR
XbaI
XbaI DIGESTION
XbaI
ITR
pAd-ΔΨ
+
ΔΨ
ITR
ITR
ΔΨ
ITR
SUPPLY OF VIRAL PROTEINS
RSV β Gal
ITR
β Gal
Ψ
ITR
RSV β Gal

FIG. 2

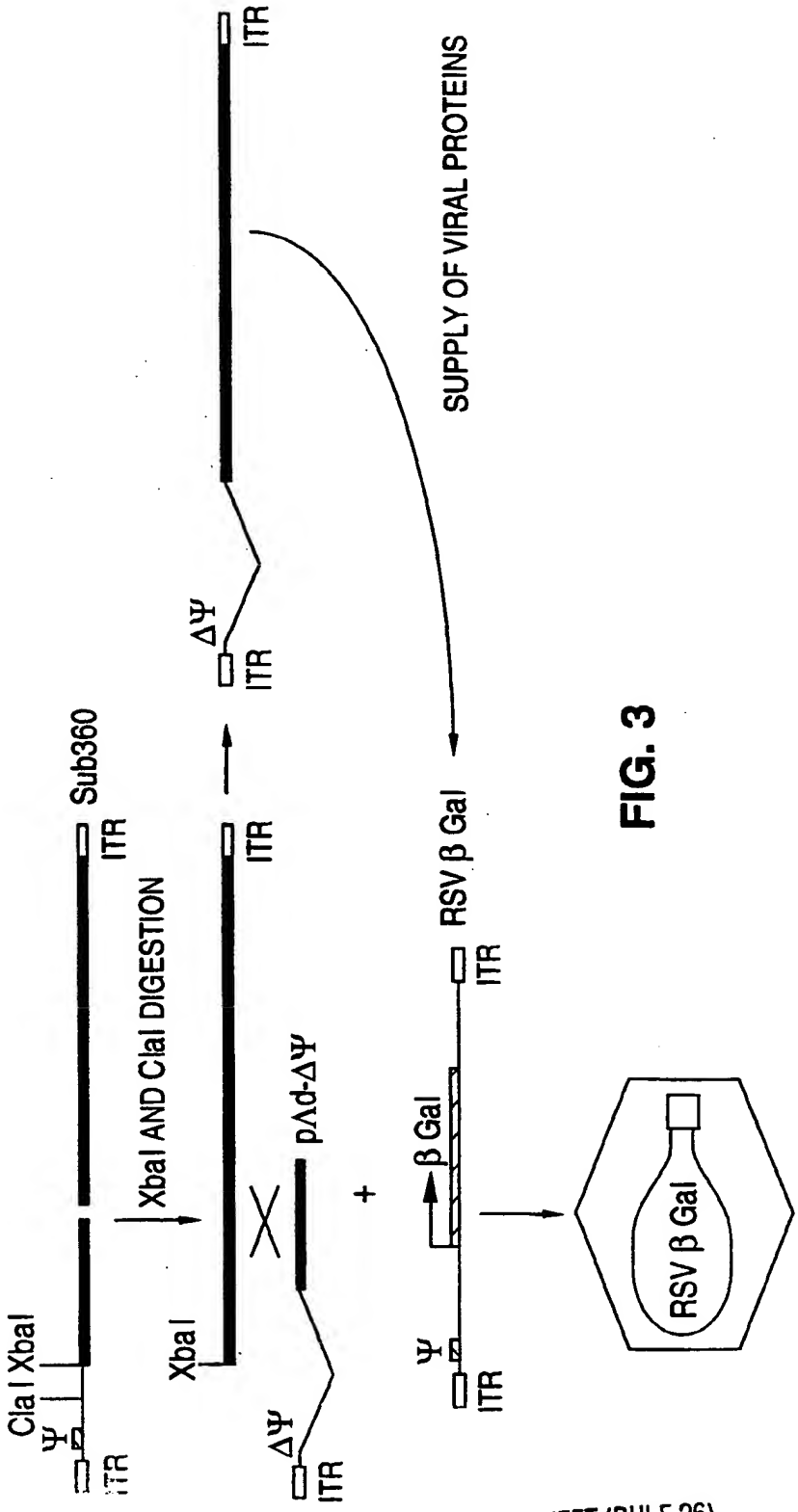
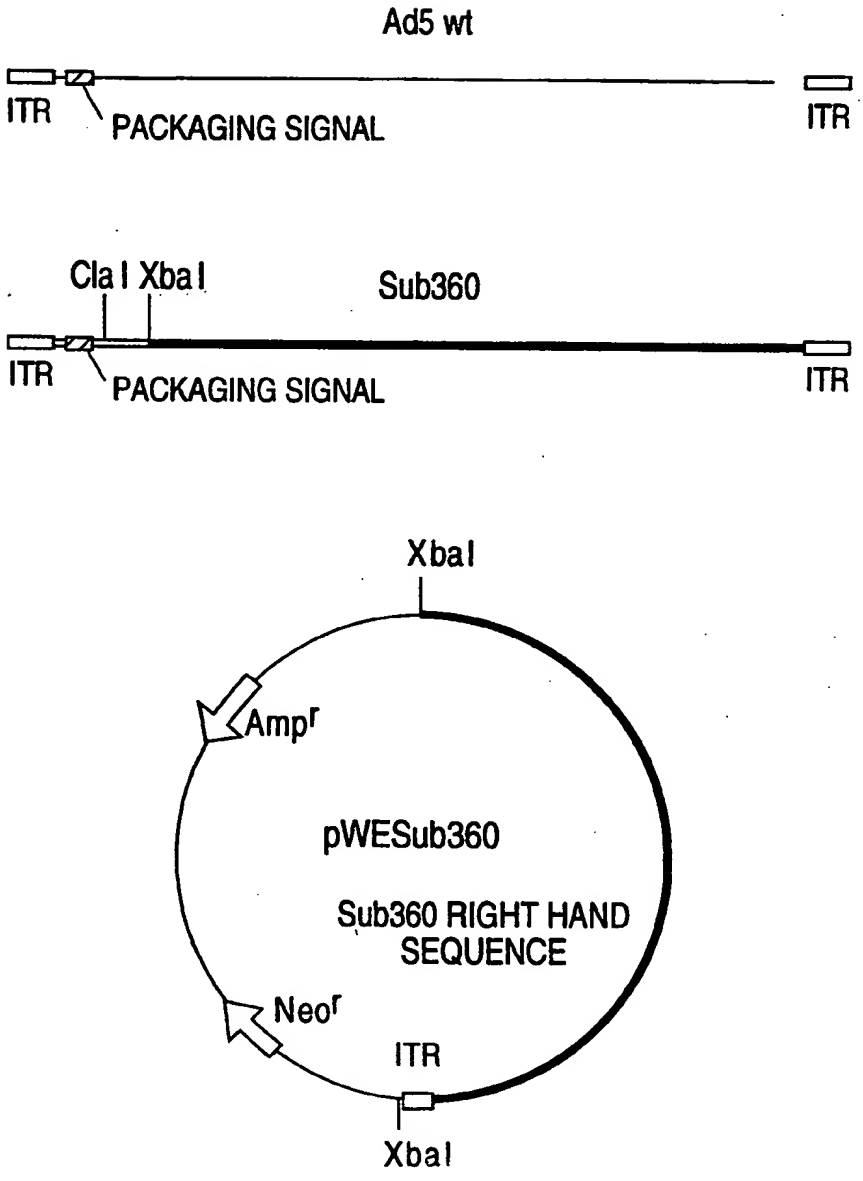
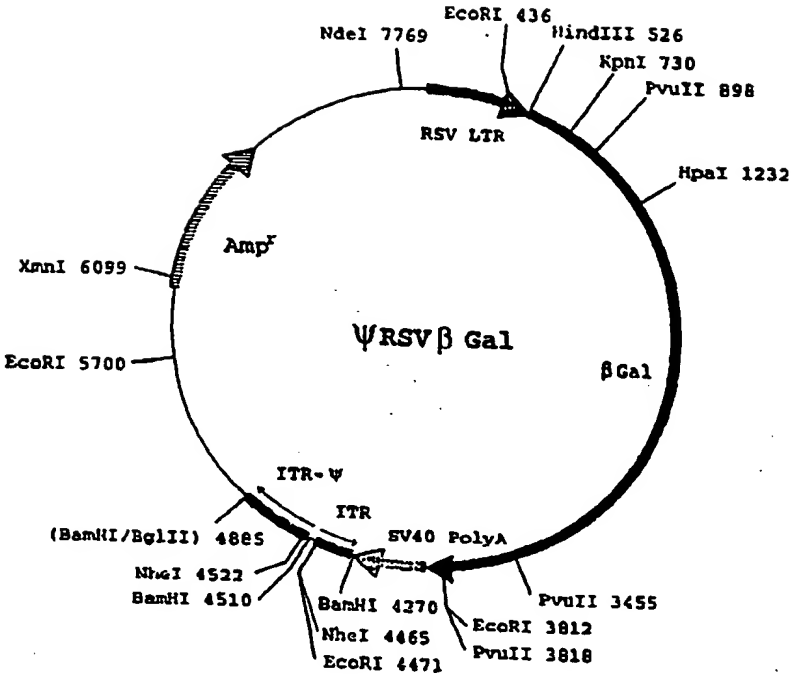

Cla I Xba I
Ψ
ITR
Sub360
ITR
Xba I AND Cla I DIGESTION
Xba I
ITR
pΔd-ΔΨ
ΔΨ
ITR
+
ITR
ΔΨ
ITR
SUPPLY OF VIRAL PROTEINS
RSV β Gal
ITR
β Gal
Ψ
ITR
RSV β Gal

FIG. 3


Ad5 wt
ITR
PACKAGING SIGNAL
ITR
Cla I Xba I
Sub360
ITR
PACKAGING SIGNAL
ITR
Xba I
Amp^r
pWESub360
Sub360 RIGHT HAND SEQUENCE
Neo^r
ITR
Xba I

FI 1

FIGURE 5


NdeI 7769
EcoRI 436
HindIII 526
KpnI 730
PvuII 898
RSV LTR
HpaI 1232
Amp^r
XmnI 6099
ΨRSVβ Gal
EcoRI 5700
βGal
ITR-Ψ
ITR
(BamHI/BglII) 4885
SV40 PolyA
NheI 4522
BamHI 4510
BamHI 4270
PvuII 3455
EcoRI 3812
NheI 4465
PvuII 3818
EcoRI 4471

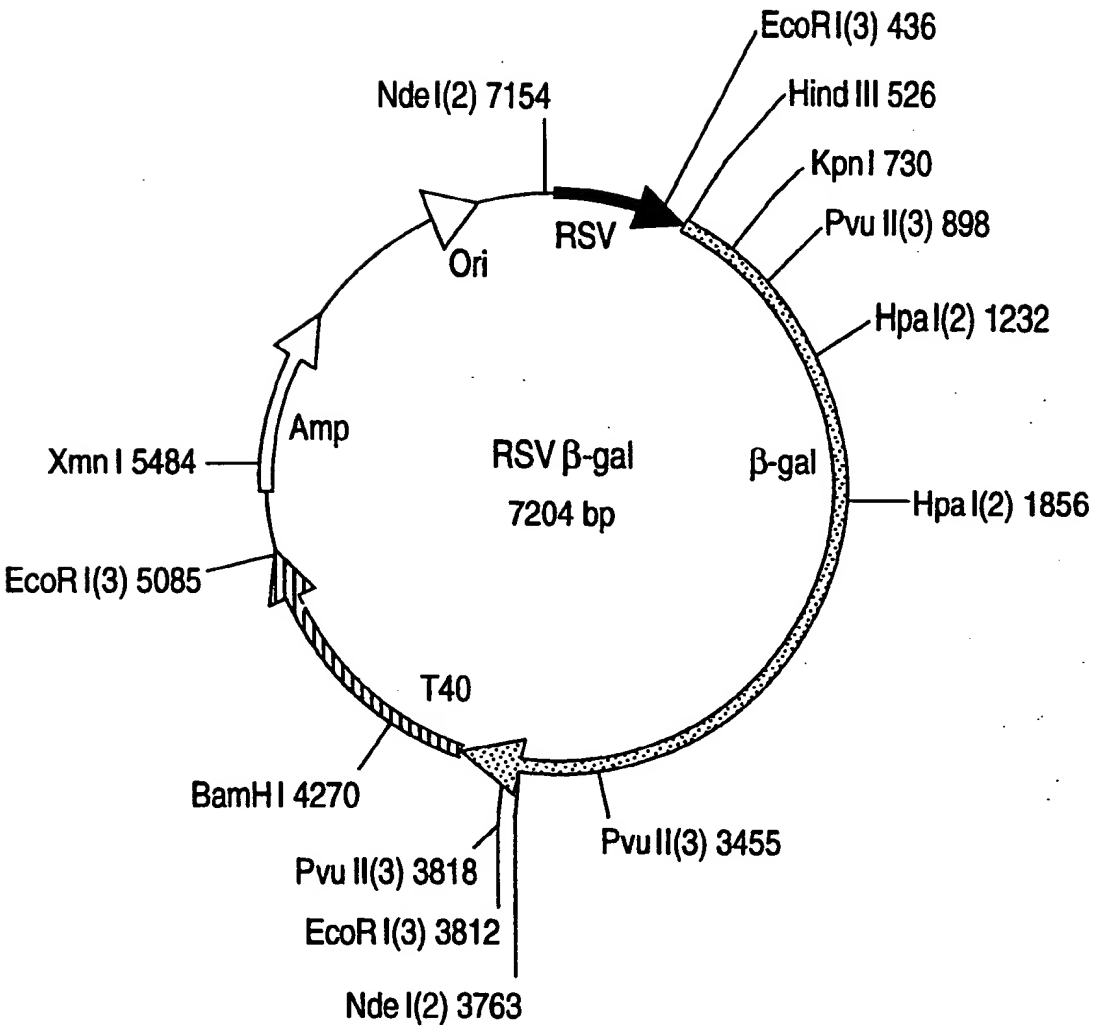

EcoR I(3) 436
Nde I(2) 7154
Hind III 526
Kpn I 730
Pvu II(3) 898
RSV
Ori
Hpa I(2) 1232
Amp
Xmn I 5484
RSV β-gal
7204 bp
β-gal
Hpa I(2) 1856
EcoR I(3) 5085
T40
BamH I 4270
Pvu II(3) 3455
Pvu II(3) 3818
EcoR I(3) 3812
Nde I(2) 3763

FIG. 6

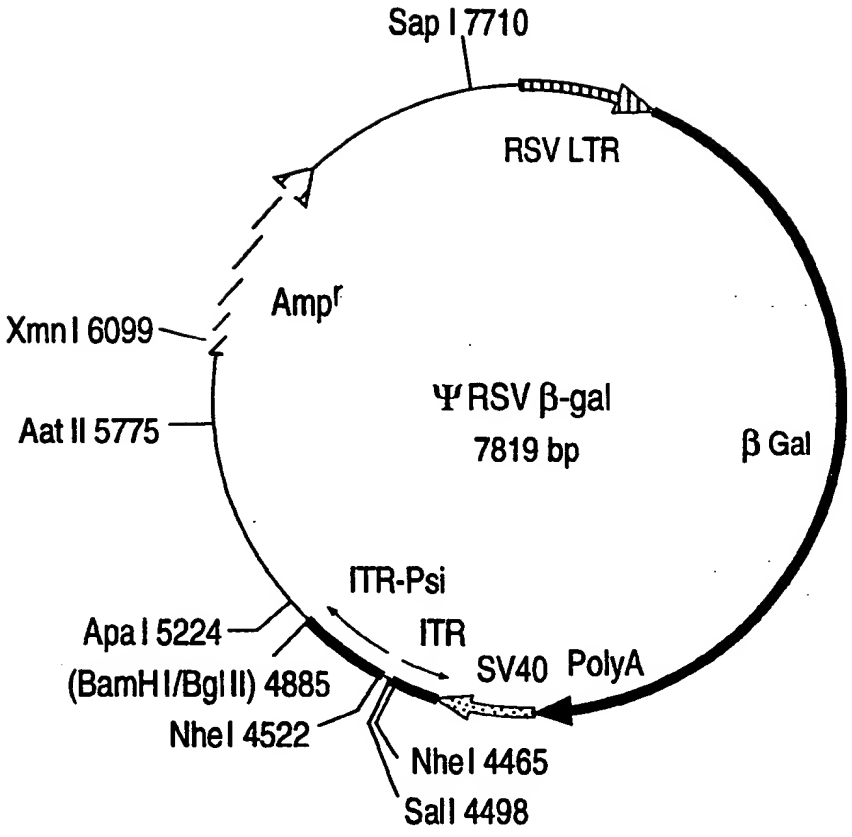

Sap I 7710
RSV LTR
Ampr
Xmn I 6099
Ψ RSV β-gal
7819 bp
β Gal
Aat II 5775
ITR-Psi
ITR
Apa I 5224
(BamH I/Bgl II) 4885
SV40 PolyA
Nhe I 4522
Nhe I 4465
Sal I 4498

FIG. 7

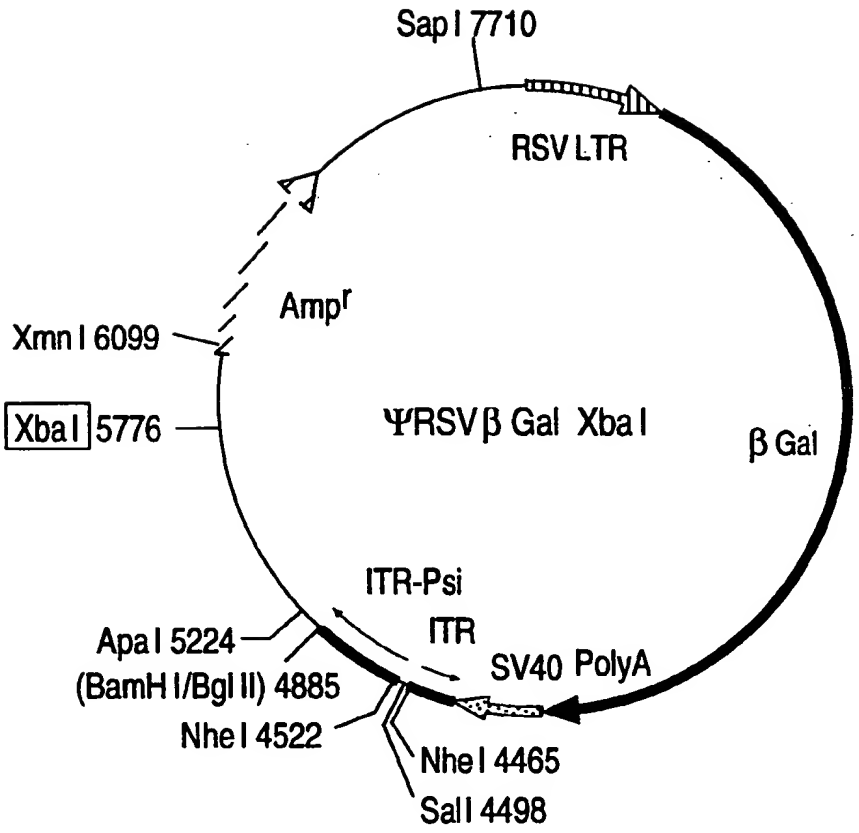

Sap I 7710
RSV LTR
Amp^r
Xmn I 6099
Xba I 5776
ΨRSVβ Gal Xba I
β Gal
ITR-Psi
ITR
Apa I 5224
(BamH I/Bgl II) 4885
SV40 PolyA
Nhe I 4522
Nhe I 4465
Sal I 4498

FIG. 8

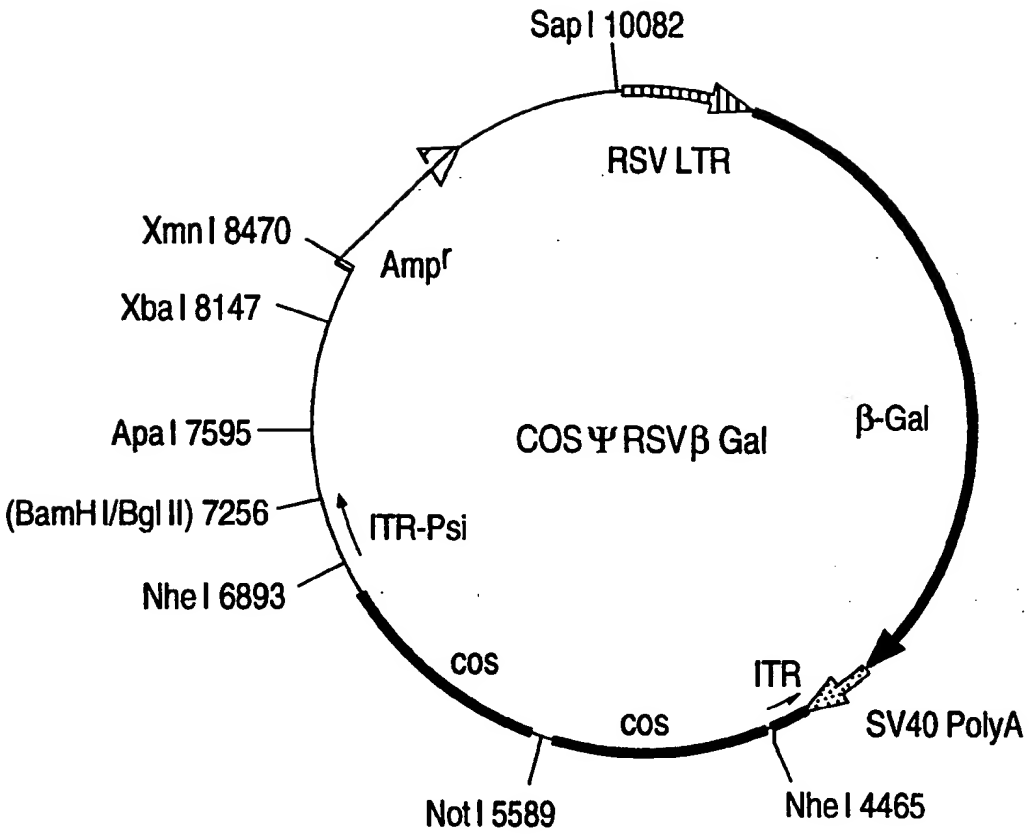

Sap I 10082
RSV LTR
Xmn I 8470
Amp^r
Xba I 8147
Apa I 7595
COS Ψ RSVβ Gal
β-Gal
(BamH I/Bgl II) 7256
ITR-Psi
Nhe I 6893
cos
ITR
cos
SV40 PolyA
Not I 5589
Nhe I 4465

FIG. 9

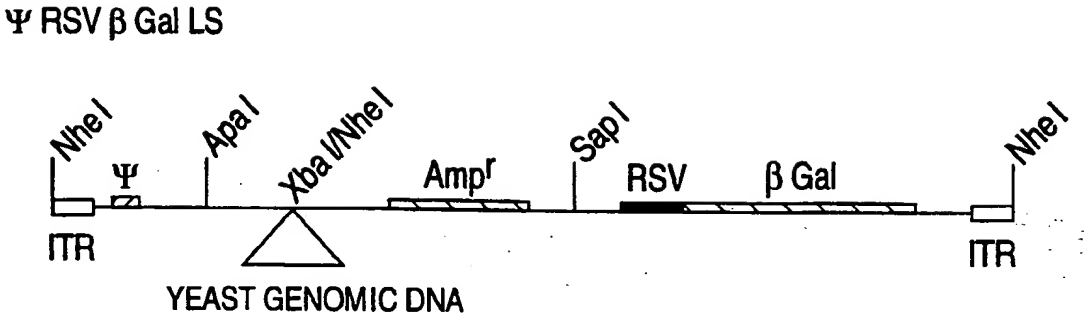
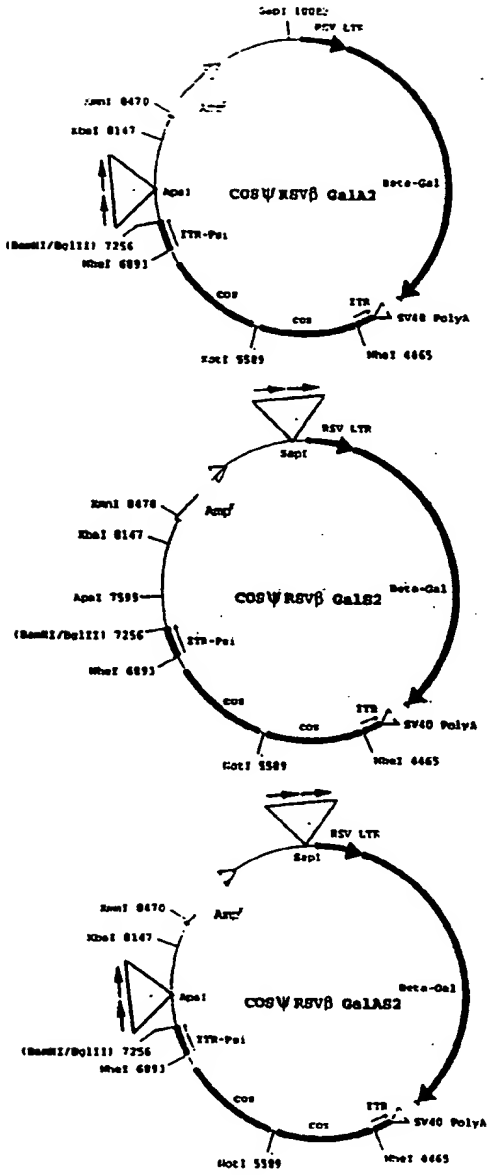

Ψ RSV β Gal LS
Nhe I
Ψ
Apa I
Xba I/Nhe I
Amp^r
Sap I
RSV
β Gal
Nhe I
ITR
ITR
YEAST GENOMIC DNA

FIG. 10

FIGURE 11


RSV LTR
XmnI 8470
XbaI 8147
ApaI
COSΨRSVβ GalA2
Beta-Gal
ITR-Psi
(BamHI/BglII) 7256
NheI 6891
cos
ITR
SV40 PolyA
NotI 5589
NheI 4465
SapI
AscI
ApaI 7595
COSΨRSVβ GalS2
COSΨRSVβ GalAS2

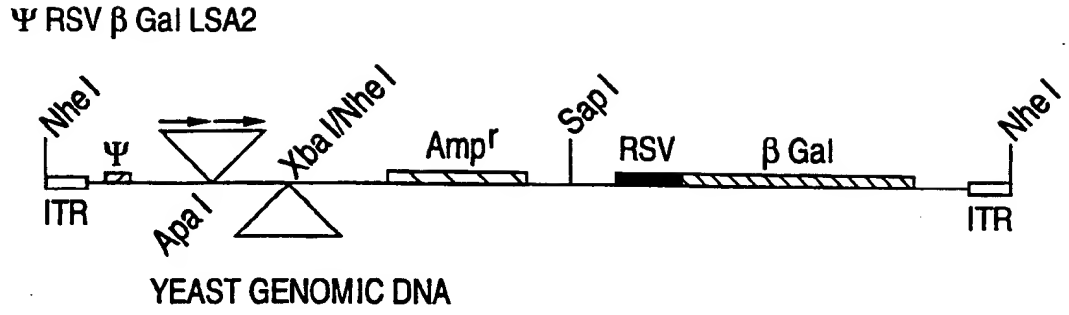


FIG. 12A

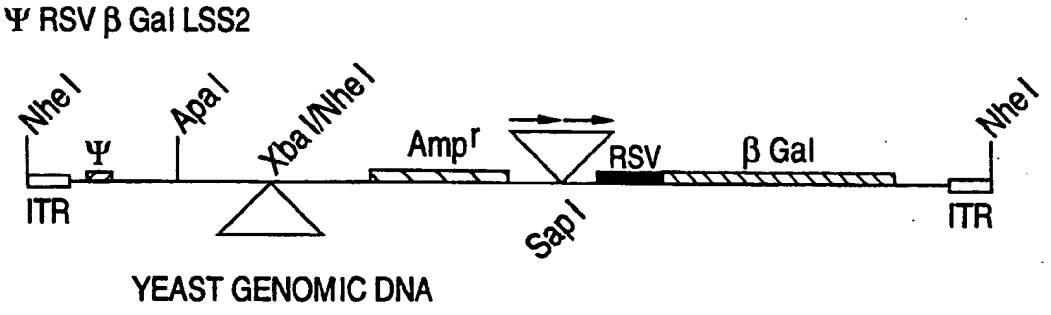


FIG. 12B

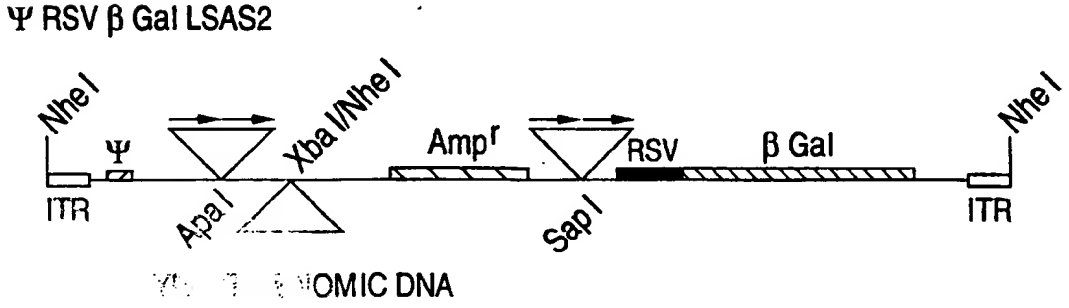


FIG. 12C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05174

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/10, 15/86
US CL : 435/172.3, 240.1, 240.2, 320.1; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 240.1, 240.2, 320.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG DATABASES: BIOSIS PREVIEWS, MEDLINE, WORLD PATENT INDEX, CA SEARCH, CAB ABSTRACTS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Proc. Natl. Acad. Sci. USA, Volume 89, issued April 1992, B. Quantin et al., "Adenovirus as an expression vector in muscle cells *in vivo*," pages 2581-2584. See entire article.	1-6, 13-30 7-12
X Y	WO, A, 93/03769 (CRYSTAL ET AL.) 04 March 1993. See entire document.	1-6, 13-30 7-12
X Y	WO, A, 94/08026 (KAHN ET AL.) 14 April 1994. See entire document.	1-6, 13-30 7-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
22 JUNE 1995

Date of mailing of the international search report
10 JUL 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
(703) 305-3230

Authorized officer
JOHNNY F. RAILEY II, PH

Telephone No. (703) 308-0

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05174

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the [illegible] Consequently, this international search report is restricted to the invention first mentioned in the claims; it i[illegible] Nos.:

Remark on Protest

☐ The additional search fees w[illegible]

☒ No protest accompani[illegible]

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05174

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-23, 29 and 30, drawn to vectors, gene expression systems, host cells comprising the vectors and methods of introducing the vectors into host cells.

Group II, claim(s) 24-28, drawn to non-human transgenic animals.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group II is a distinct invention, not necessarily derived by using the vectors of Group I. In addition, the vectors of Group I are used to generate the transduced host cells also found in Group I. Group II is a separate and distinct use of the vectors of Group I.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.